

2012 International Conference on Medical Physics and Biomedical Engineering

Construction of Expression Vector for Anti-Alpha-Fetoprotein Gene and Its Inhibition Effects on Alpha-Fetoprotein Positive Hepg2 Cells

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Abstract

As research previously demonstrated, suppression of AFP expression or its biological activities might inhibit the proliferation of AFP positive human hepatocellular carcinoma cells. In this study, we constructed an anti-AFP gene vector and transfected it to HepG2 cells. RT-PCR showed AFP gene expression in the transfected cells was reduced. MTT assay suggested the proliferation of the transfected cells was also inhibited comparing with the untransfected cells. This result provides a new insight into AFP as the target for preventing and treating hepatocellular carcinoma.

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Keywords: Hepatocellular carcinoma; Alpha-fetoprotein; HepG2

1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumor worldwide. It is one of the leading causes of malignancy-related death in China. A number of strategies such as surgery, radiation, chemotherapy, and biological response modifiers have been applied for the treatment of HCC. However, there is still no satisfactory method to cure the disease [1].

With the emerging revolution in cellular signaling transduction technology, more and more biological methods are used in treatment of cancer. Alpha-fetoprotein (AFP) is an oncoembryonal protein with multiple cell growth regulating, differentiating and immunosuppressive activities. It has been well characterized as a physiological carrier/transport protein for various ligands [2-5]. As a diagnostic tumor-specific marker of hepatocellular carcinoma [6], AFP can suppress the immune system and help hepatocarcinoma cells escape from immune surveillance. Besides, the relationship between AFP and HCC is far from clear. Recent investigations indicate that human AFP can enhance the proliferation of mouse

hepatoma H-22 and human hepatoma SMMC-7721, BEL-7404 or QGY-7703 cells *in vitro* [7,8]. Similar growth stimulatory effect of low concentrations of AFP has been obtained in human hepatoma HepG2 cells. These results have an important implication that suppression of AFP expression or its biological activities may be a new strategy for the treatment of AFP-associated tumors such as HCC. AFP should be a good target in gene therapy for HCC.

Antisense oligodeoxynucleotides can prevent mRNA translation by forming RNA-DNA duplexes and therefore, the inhibition effect of antisense oligodeoxynucleotides is in transcription level. Now antisense oligodeoxynucleotides technique has been commonly used to investigate their role in the treatment of human malignancies, both *ex vivo* and *in vivo* [10, 11]. Moreover, in our previous research, we found that AFP-specific transfer factors could inhibit the growth of AFP positive HepG2 cells through downregulate AFP expression in post-transcription level [9]. So in this report, we constructed a recombinant antisense-AFP gene vector named pEGFP-anti-AFP and transfected the vector to HepG2 cells. Then cell viability was detected by MTT assay. The results suggest that the proliferation of cells transfected with antisense-AFP gene was evidently inhibited.

2. Materials and methods

2.1 Cell lines

Hepatocarcinoma cell line HepG2 (AFP positive) were preserved in our laboratory. Cells were cultured in DMEM medium (Gibco) containing 10% fetal calf serum (Hyclone), 100 IU/ml penicillin and 100 µg/ml streptomycin sulfate (Amersco) at 37°C in saturated humidified air with 5% CO₂. The cells were subcultured every two days.

2.2 Reagents, bacterial strains and plasmids

Enzymes and Reagents DNA restriction endonuclease; Taq DNA polymerase; T₄ DNA Ligase; DNA isolation and Purification kit (Dalian TakaRa Biotechnology, China)

Bacterium and Vector *Escherichia coli* DH5, pMD18-T Vector and pEGFP-N1 vector were conserved by laboratory. pCMV-SPORT6-AFP that bears the whole cDNA of AFP gene was purchased from Wuhan Sanying Biotechnology.

Subcloning of antisense-AFP gene PCR was used to amplify the antisense-AFP cDNA region of pCMV-SPORT6-AFP. In order to introduce *Xho* I and *Eco*R I sites into the sense and antisense chains respectively, a pair of PCR primers was designed using computer software (Primer Premier 5.0) as follows: *Xho*-AFP-5': 5' tc ctc gag gca acc atg aag tgg gtg gaa t 3' (sense), *Eco*-AFP-3': 5' gt gaa ttc g aac tcc caa agc agc acg agt 3' (antisense). PCR was performed in a total volume of 50 µl consisting of 5 µM each primer, 2.5mM each dNTP, 5 µl 10×polymerase reaction buffer, 2.5U Pfu DNA polymerase and 2 µl DNA template. The samples were heated to 94°C for 3 min followed by amplification for 35 cycles at 94°C for 1min, 58°C for 1min, and 72°C for 3.5min. After the last cycle, a final extension step was at 72°C for 5 min. Then 5 µl of each product was analyzed by 1% agarose gel electrophoresis. PCR products were purified from the agarose gel using DNA purification kit.

Construction of recombinant plasmid After clean-up and A-tailing reaction with Pfu/A Amp Tailing Kit (Dalian TakaRa), PCR products of anti-AFP cDNA region and pMD18-T Vector were ligated with T₄ DNA Ligase, then the new plasmid, named pMD18-antisense-AFP, was transformed to DH5 cells to amplify. The pMD18-antisense-AFP and pEGFP-N1 were both doubly digested with *Xho* I and *Eco*R I. CIAP in this reaction system was used to dephosphorylate. We can obtain the full-length of antisense-AFP and pEGFP-N1 after the digested products were purified from the agarose gel using DNA

purification kit. Then the full-length antisense-AFP identified with *Xho* I and *Eco*R I cleavage was subcloned into expression vector pEGFP-N1. The recombinant plasmid was identified with restriction endonuclease *Xho* I and *Eco*R I cleavage, PCR amplification. Then it was confirmed by sequencing in TaKaRa. The obtained sequence data were compared with cDNA sequence from GenBank (Accession No. NM001168). Finally, the recombinant plasmid was transformed to DH5 cells to amplify.

2.3 Transfection and identification of pEGFP-antisense-AFP to HepG2 cells

HepG2 cell monolayers in a 96-well plate at a density of 1×10^4 /well were transfected with 1–2 μ g per well of pEGFP-antisense-AFP by using 2–4 μ l LipofectamineTM 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

RT-PCR was used to detect the expression of AFP gene in the transfected HepG2 cells. Briefly, total RNA of detected HepG2 cells was isolated using TRIzol reagent (Invitrogen). About 1 μ g of total RNA was used for first-strand cDNA synthesis with a kit according to the manufacturer's instructions (Invitrogen). Then cDNAs were amplified with the following primer pairs: AFP, 5'-AAAAGCCCACTCCAGCATC-3' and 5'-CAATCCAGCACATCTCCTC-3'; β -actin was used as the control. The PCR products were separated on ethidium bromide stained agarose, and visualized with UV. Then we got the different expression of AFP gene in the detected HepG2 cells and validated successful transfection.

Cells viability assay After transfected cells were planted in 96-well cell culture plates (Costar, Corning, NY) for 24 h, Cell viability was determined by MTT assay [12].

Statistical Analysis The results were expressed as the mean \pm SE accompanied by the number of experiments performed independently.

3. Results

3.1 Subcloning of antisense-AFP gene

Figure 1 shows that antisense-AFP gene is successfully amplified.

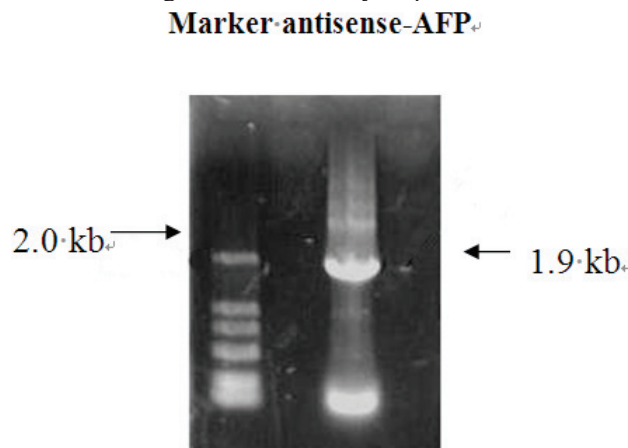


Fig.1 Subcloning of antisense-AFP gene

3.2 Construction of recombinant plasmid

Fig.2 and Fig.3 indicate that pEGFP-antisense-AFP recombinant plasmid is successfully constructed.

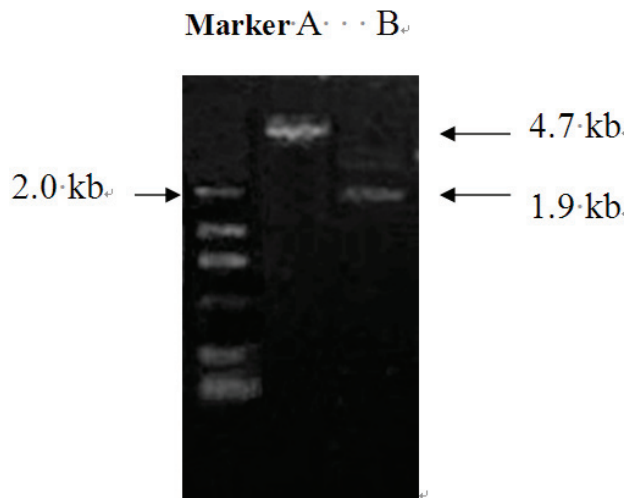


Fig.2 Digestion of pEGFP-N1 (A) and pMD18-antisense-AFP (B) with Xho I and EcoR I

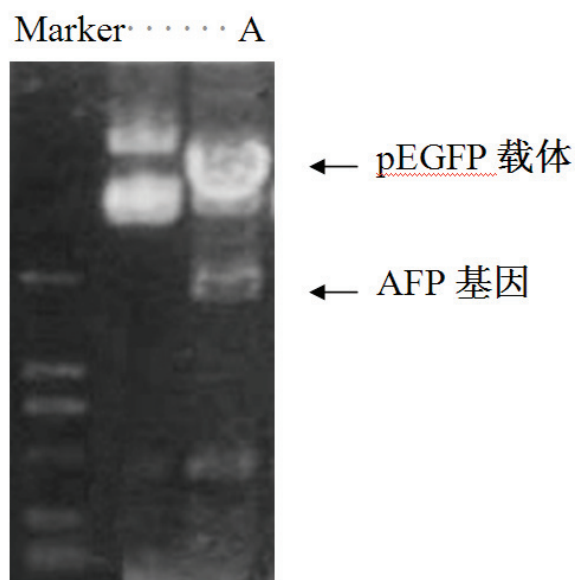


Fig.3 Digestion of pEGFP-antisense-AFP (A) with Xho I and EcoR I

3.3 Identification of pEGFP-antisense-AFP transfection in HepG2 cells

Our study shows the best transfection efficiency is realized when pEGFP-antisense-AFP/LipofectamineTM 2000 is 1/2. RT-PCR shows the expression of AFP gene is decreased after transfection with pEGFP-antisense-AFP for 24h comparing with untransfected cells.

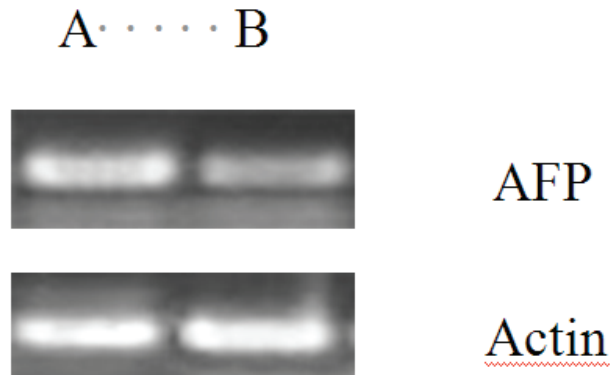


Fig.4 RT-PCR shows AFP gene expression in the normal HepG2 cells (A) and the transfected HepG2 cells (B)

3.4 Cells viability analysis

After pEGFP-antisense-AFP vector transfected HepG2 cells for 24h, cell viability decreases to 78.13%.

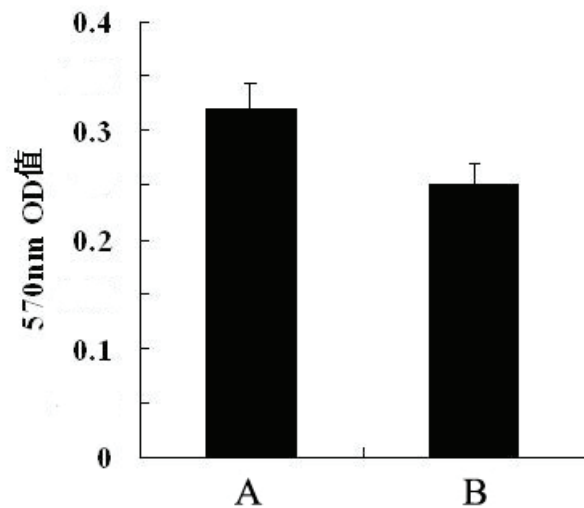


Fig.5 MTT assay for normal HepG2 cells (A) and transfected HepG2 cells (B)

4. Discussion

In recent years, the growth regulatory properties of AFP have aroused our interests. Studies involving ontogenetic and oncogenic growth in both cell cultures and animal models have been ongoing. Particularly, AFP can regulate the expression of some oncogene and cancer suppressor gene such as c-fos, c-jun, and N-ras [13]. Therefore, AFP has become the target gene in many investigations. Wang YS constructed AFP siRNAs expressing plasmid and studied the effect of AFP on hepatoma cells. They found treatment of SMMC-7721 cells with AFP siRNAs could inhibit the growth of SMMC-7721 [14]. Wang XW showed that AFP antisense phosphorothioate oligodeoxynucleotides effectively inhibited the growth of Bel7404 human hepatoma cells in vitro [15]. His another experiment indicated AFP antisense phosphorothioate oligodeoxynucleotides could inhibit tumour growth in Balb-c nude mice bearing SMMC-7721 tumors [16].

We bought the pCMV-SPORT6 plasmid containing antisense-AFP gene and subcloned antisense-AFP gene. A pair of PCR primers was designed to introduce *Xho* and *Eco*R1 sites. PCR products were ligated with pMD18-T Vector. It was used to construct the recombinant antisense-AFP gene vector named pEGFP-antisense-AFP through connecting with the plasmid of pEGFP-N1 after both doubly digested with *Xho* and *Eco*RI. Then we transfected the pEGFP-antisense-AFP to HepG2 cells by Lipofectamine 2000. RT-PCR suggested that the expression of AFP gene was reduced in the transfected cells comparing with untransfected cells. Furthermore, we analyzed the growth activity of transfected and untransfected cells using MTT assay. We found that the growth of transfected cells had been inhibited.

In our previous study, we use AFP as the antigen to prepare AFP-specific transfer factors (AFP-TF) and found for the first time that AFP-TF could inhibit the growth of Bel7402 and HepG2 AFP positive hepatocarcinoma cells, and induce their apoptosis [9]. However, we don't find the apoptosis of transfected HepG2 cells in this research. So we consider that the downregulation of AFP expression induces growth inhibition of HepG2 cells, but not the apoptosis.

In conclusion, the present results indicate that AFP antisense phosphorothioate oligodeoxynucleotides expressing plasmid exhibits significant antihepatoma activities *in vitro*. However, the mechanisms of its antihepatoma action are not through the induction of hepatoma cell apoptosis.

Acknowledgments

This work was supported by the Dean's fund(201-52011005) and Doctor Startup Fund of Shenyang Normal University(054-55440107029).

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